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Downregulation of CD43 in RAEB and RAEB-T Patients. Report of 3 Cases

D.S. Kyriakou,[1]* M. Alexandrakis,[1] M. Tzardi,[2] D. Stephanaki,[2] and G.D. Eliopoulos

[1]Department of Hematology, University Hospital of Herakleion, Crete, Greece
[2]Department of Pathology, University Hospital of Herakleion, Crete, Greece

CD43 (leukosialin, sialophorin) is a cell surface mucin expressed at high levels on most leukocytes and is reported to be involved in adhesion, anti-adhesion, and signal transduction prodders. Regulation of its expression is thought to take place through methylation of the DNA in the nonproducing cells, and the methylation inhibitor 5-azacytidine induces expression of the sialophorin gene. Here we report three cases of patients with myelodysplastic syndromes in which acquired severe deficiency of the CD43 antigen on the surface of most hemopoietic cells was observed. Peripheral blood mononuclear (PBMC) cells from 32 MDS patients and 20 healthy individuals were analyzed by flow cytometry after labeling with an anti-CD43 (DF-T1) monoclonal antibody. In 1 patient with refractory anemia with excess of blasts (RAEB) and 2 patients with refractory anemia with excess of blasts in transformation (RAEB-t), the percentages of CD43(+) PBMC were 3.8%, 6%, and 9.9%, respectively. The deficiency was observed at protein and RNA level as confirmed by western and southern blot, while analysis of the DNA by single-strand conformation polymorphism and sequencing did not reveal any difference in the gene sequence between the CD43(+) and CD43(−) cells of these patients. It is known that patients with MDS may have normal and dysplastic population of hemopoietic cells. Further studies are needed to reveal the mechanism of downregulation of the gene in these 3 patients and whether the phenomenon is related to the dysplastic population only or not. Am. J. Hematol. 63:20–27, 2000.

Key words: CD43; sialophorin; myelodysplastic syndromes

INTRODUCTION

CD43 is a heavily glycosylated transmembrane molecule that is thought to possess pro-adhesive and anti-adhesive properties and is expressed on most leukocytes and platelets [1,2]. Multiple glycoforms of CD43 are generated by glycosyltransferase-mediated alterations in the O-glycans attached to the protein core [1]. The predominant CD43 glycoform of the resting T-lymphocytes is CD43 (115 kDa), while the peripheral T-cell activation antigen is CD43 (130 kDa) [1,2]. It is believed that the dense negative charge of its extracellular domain provide a barrier around the cells resulting in diminished cell–cell interactions [3–6]. On the other hand, it has been shown to transmit signals leading to increased avidity of other adhesion molecules acting in a pro-adhesive manner [7–12]. Regulatory signals are reported to be transduced through CD43, as suggested by the ability of anti-CD43 antibodies to induce aggregation and proliferation of T-cells and to enhance B-cell proliferation and natural killer cell activity. There is evidence that it may act as a substrate of protein kinases during the mitogenic activation of lymphocytes [13,14].

It has been published that the leukosialin gene is downregulated in nonproducing cells by DNA methylation. The leukosialin promoter has an Sp1 binding site and a sequence similar to an initiator [15]. The DNA methyltransferase inhibitor 5-azacytidine was able to induce expression of the gene in non-expressing cells [15].

Here we present 3 rare cases with MDS where the peripheral blood and bone marrow mononuclear cells expressed very low amounts of CD43 at protein and

*Correspondence to: Despina Kyriakou, MD, PhD, Assistant Professor of Hematology, University Hospital of Herakleion, P.O. Box 1352, Herakleion, Crete, Greece.

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TABLE I. Some Clinical Features of the Patients Studied*

Characteristic	RA	RAS	RAEB	RAEB-t	CMML
No Pts	14	3	6	3	3
Sex					
F	3	2	1	1	1
M	11	1	5	2	2
Age (years)					
F	72	59	76	–	87
M	67.7	71	73.2	63	77
Duration of disease (months)	43.3	105	30.7	15	54.6
Hepatomegaly				1	2
Splenomegaly	5	0	1	0	3
Lymphadenopathy	0	0	0	0	0
Hb (g/dl)	9.8 ± 0.6	8.3 ± 0.3	9.3 ± 1.5	8 ± 0.1	6.8 ± 0.8
Leukocytes × 10^3 (/μl)	2.27	4.8	3.27	3.45	27.17
Neutrophils	2,185 ± 1,130	3,160 ± 1,350	1,521 ± 658	1,130 ± 817	2,518 ± 1,159
Lymphocytes	2,986 ± 998	2,796 ± 802	3,210 ± 452	2,085 ± 1,120	3,960 ± 2,147
Monocytes	423 ± 285	412 ± 147	321 ± 189	622 ± 269	1,895 ± 1,119
Blasts	0	0	128 ± 189	695 ± 528	421 ± 282
Platelets × 10^3 (/μl)	118 ± 59	298 ± 90	95 ± 30	68 ± 45	102 ± 58
Bone marrow					
Hypocellular	1	0	0	0	0
Hypercellular	12	3	6	3	3
Fibrosis	1	0	0	0	0
Autoimmune phenomena	0	0	0	0	0
Autoantibodies	7	0	2	0	2
CRP	4	0	2	1	1
RF	3	1	2	0	1
HBV	5	0	0	0	0
HCV	1	0	0	0	0
CMV	4	0	1	1	0
EBV	6	0	2	1	0
Chromosome abnormalities			1	1	

*All values are expressed as means ± 1 SD. Age is expressed as median. Autoantibodies positive were antinuclear antibodies, anti-DNA antibodies, anticardiolipin antibodies.

RNA levels raising the suggestion that this gene is probably downregulated in an acquired manner in these cases. The factors that are involved in this phenomenon remain to be studied.

PATIENTS, MATERIALS, AND METHODS

Patients

Thirty-two patients with myelodysplastic syndromes were studied. Twenty-three were men of median age 69 (range 60–83) and 9 were women with median age 71 years (range 33–87). Twelve were diagnosed as refractory anemia (RA), 3 as refractory anemia with ringed sideroblasts (RAS), 6 as refractory anemia with excess of blasts (RAEB), 2 as refractory anemia with excess of blasts in transformation (RAEB-t), and 2 as chronic myelomonocytic leukemia (CMML).vThe diagnosis was based on the criteria proposed by the French-American-British Cooperative Group (FAB classification) [16].

The duration of the disease before the study was 3.5–108 months with a median of 40.5 months. None of the patients had previously received any kind of treatment for their disease except of occasional red blood cell and/or platelet transfusions. Some clinical and laboratory features of the patients are shown in Table I.

Isolation of Peripheral Blood and Bone Marrow Mononuclear Cells

Peripheral blood (PBMNC) and bone marrow (BMMNC) low-density mononuclear cells were isolated following standard centrifugation on Ficoll-Hypaque (lymphoprep 1.077 g/ml, Nycomed, Oslo Norway) and two washes with Hank's balanced solution (HBSS) supplemented with 0.2% bovine serum albumin (BSA) (Sigma, St. Louis, MO) as previously described [17].

Flow Cytometric Analysis

Flow cytometric studies were performed on whole blood and whole bone marrow using the erythrocyte lysing method as previously described [17]. Mononuclear cells isolated as described above and leukocytes after erythrocyte lysing were labeled with an anti-CD43, FITC conjugated, monoclonal antibody recognizing the 115-kDa isoform of the antigen (clone DF-T1, Serotec). The

cells were also stained with the following phycoerythrin conjugated monoclonal antibodies: anti-CD14, -CD33, -CD34 (Q-Bend10), -CD45, -CD56. All the antibodies were obtained from Immunotech (Marseilles, France). The method used for labeling has been described earlier [17]. Especially before incubation with the monoclonal antibodies the cells were incubated with 100 μl of normal human serum as blocking reagent. After labeling, the cells were fixed with the Q-Prep reagent system (Coulter, U.K.) according to the manufacturer's instructions. An isotype-matched mouse IgG FITC or Phycoerythrin conjugated was used for parallel labeling and served as control for subtraction of the background (amplification and compensation settings of the flow cytometer).

The cells were analyzed on an Epics Elite fluorescence flow cytometer and were gated according to the light scattering properties at 0 and 90°. The percentage of the single-positive and double-positive cells were measured, and the mean channel value (MCV) corresponding to the mean fluorescence intensity (MFI) was estimated. After CD43(+) and CD43(−) labeling, cells were separated using a rabbit anti-mouse monoclonal antibody conjugated with magnetic beads and a magnetic supporter (Immunotech).The purity of CD43- cells was 100% as it was checked by flow cytometry and the purity of CD43+ cells reached 95%. CD34+ cells were isolated after labeling with Q-bend10 anti-CD34 monoclonal antibody and incubation with magnetic beads-conjugated antibody recognizing Q-bend10 [18,19]. The mini Macs CD34 cell isolation kit was used according to the manufacturer's instructions (Miltenyi Biotech Sunnyvale, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for mRNA Expression of Sialophorin Gene

RNA extraction from bone marrow mononuclear cells, PBMNC, CD43+ cells, CD43− cells, and CD34+ cells were done with TRIZOL LS (Gibco-BRL) reagent according to the manufacturer's instructions. For reverse transcription the superscript kit (Gibco-BRL) was used according to the manufacturer's instructions with oligo dT-priming. PCR was performed at 94°C for 1 min, 60°C for 20 s, and 72°C for 20 s for 35 cycles after an initial denaturation step at 94°C for 2 min with the following specific sets of primers: CD43-specific primers (CD43 F2), 5′-gcg-tgt-tct-gct-tct-ccg-gct-gcc-3′; CD43R2; 5′-gag-act-cag-cag-ccg-ttt-cca-agg-a-3′; for nested pcr CD43F4, 5′-att ggt gcc agc act ggt tcc c-3′; CD43R1, 5′-tct gga gag ttc gtt gtt atg g-3′ giving a PCR product of 181 bp within the second intron of the gene. B2-microglobulin-specific primers: 5′B-2, 5′-atg-tct-cgc-tcc-gtg-gcc-tta-gct-3′; 3′B-2, 5′cct-cca-tga-tgc-tgc-tta-cat-gtc-3′ giving a PCR product of 370 bp.

Northern Blot Analysis

Thirty micrograms of total RNA from PBMNC and BMMNC, isolated CD43(+) and CD43(−) cells were applied to 1.2% agarose gels containing 6.7% formaldehyde and electrophoresed. RNA was transferred to a nylon membrane filter (Bio-Rad) in 20× SSC. Hybridization was performed at 65°C overnight with a DNA probe obtained by PCR with CD43F4-CD43R2 primer pair and was labeled by PCR with $dATP^{32}$ as previously described [20]. The hybridization buffer contained 2× SSPE, 10× Denhardt's solution, 2% SDS, and 100 mg/ml denatured salmon sperm DNA. The filter was first washed in 2× SSC and 0.05% SDS at room temperature for 40 min and then in 0.1% SSC and 0.1% SDS at 65°C for 20 min twice.

Western Blot Analysis

Western blot analysis was performed as described previously [21]. PBMNC and BMMNC as well as CD43(+) and CD43(−) total bone marrow cells and CD34+ cells were isolated as described above, washed with phosphate-buffered saline (PBS) and lysed with Laemmli's sample buffer. The cell lysates from 10^5 cells were boiled for 5 min, and the protein content was measured with the Coomassie blue method. Equal amount of protein was applied to the lanes in the 12% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to nitrocellulose filters and probed with mouse anti-human anti-CD43 (DFT-1) antibody after blocking with normal bovine serum. A horseradish peroxidase conjugated secondary antibody was used and the complex was detected with ECL kit (Amersham, Little Chalfont, U.K.) and by DAB method.

Mutation Analysis of the Sialophorin Gene

DNA extraction. DNA from total BMMNC and PBMNC was extracted using standard phenol/chlorophorm/isoamyl alcohol method as described before [23].

Single strand conformation polymorhism (SSCP). SSCP was performed as described earlier [24]. Polyacrylamide gels 12% (37:1;Gibco-BRL) were used in a discontinuous electrophoresis system. The gel buffer was 60 mmol/l formic acid adjusted to pH 9.0 with Tris and PEG 5 g/L. The trailing buffer used was Tris-glycine (0.5 mol/l Tris and 50 mmol/l glycine), pH 8.3.

Overlapping PCR products 300–500 bp long obtained from DNA of CD43+ and CD43- BMMNC of the 3 patients were loaded to the gels after initial denaturation to 96°C for 4 min and immediate chilling on ice. The amount of DNA loaded was 500–800 ng. The sample buffer was 980 ml/l formamide,10 mmol/l EDTA, 0.25 g/l xylene cyanol FF, and 0.25 g/l bromophenol blue. The gels were run at 5 W overnight at 4, 18, and 37°C. After

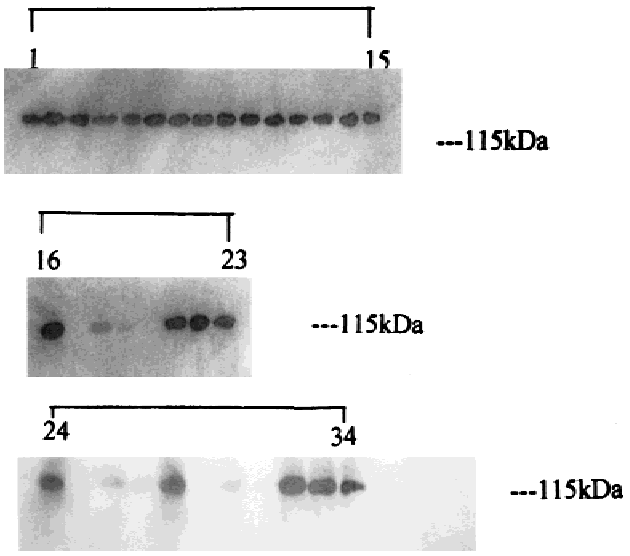

Fig. 1. Western blot analysis of cell membranes of bone marrow mononuclear cells (BMMNC) from patients with MDS. The filters were hybridized with DF-T1 anti-CD43 antibody recognizing the 115-kDa isoform of the molecule. Lanes 1–15, 21–23, and 32–34: BMMC from patients without CD43(−) population. Lane 16: CD43+ BMMC from patient A after magnetic separation. Lane 17: CD43(−) BMMNC from patient A after magnetic separation. Lane 18: Total BMMNC. Lane 19: Total CD34(+) cells from bone marrow of the patient A. Equal amount of protein was loaded to all lanes as it was determined with Coomassie blue micromethod. Lanes 24–27: CD43(+), CD43(−), and total BMMC and total CD34(+) BMMNC respectively from patient B. Lanes 28–31: CD43(+), CD43(−), and total BMMNC and total CD34(+) BMMNC respectively from patient C.

completion of the electrophoresis the gels were silver stained [24].

DNA sequence analysis. DNA sequence was performed on a Pharmacia α express sequenator (neon laser) with the dye primer method (CWS-primer) and the assembly was done on the GCG program.

Immunohistochemical Analysis

Bone marrow trephine biopsies were proceeded for immunohistochemistry as described before [25]. After deparaffinization and rehydration in TBS the specimens were first incubated with human normal serum to block unspecific binding, then they were exposed to anti-CD43 mouse monoclonal antibody for 1 h and then to APAAP complex for 30 min. Naphthol AS-MX phosphate with Fast Red TR salt and levamisole were used as substrates.

Aggregation Assay

Quantitative aggregation assays were performed in 96-well tissue culture plates (Costar, Cambridge, MA). Cells were plated at 5×10^5/ml in RPMI-1640+10% fetal calf serum and stimulated with the anti CD43 mAb DF-T1 at 10 μg/ml in a total reaction volume of 100 μl for 72 h at

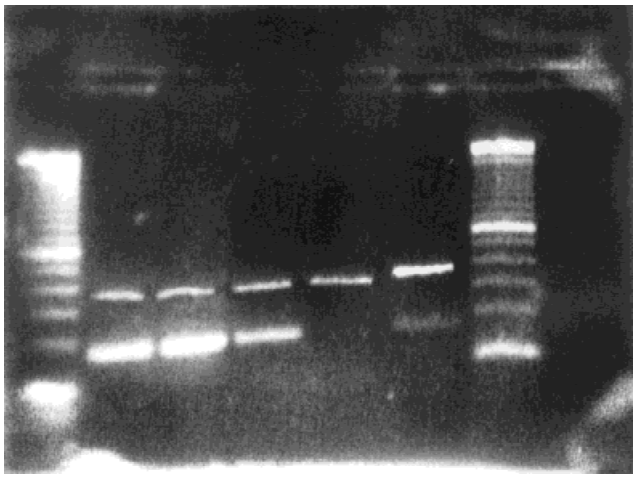

Fig. 2. RT-PCR from patient A. Lanes 1 and 7: 100 bp DNA ladder. Lanes 2 and 3: Two RA patients without CD43(−) population in bone marrow. Lane 4: CD43(+) BMMNC isolated with magnetic separation from patient A. Lane 5: CD43(−) cells from BMMNC after magnetic depletion. Lane 6: CD34(+) BMMNC. The CD43 product is of 187 bp size and the b2-microglobulin gene amplified simultaneously is of 370 bp size. The relative intensity of the CD43/b2-m bands were for lanes 2–6: 2.75, 2.83, 2.092, 0.073, and 0.278, respectively.

37°C. The incubation was done before and after incubation with anti-CD11a blocking antibody. Suspentions of each sample were mixed by pipetting up and down 20 times and transferred to a Neubauer counting chamber, where the number of free cells was assessed. Percentage of aggregation was determined as (1 − number of free cells/original cell number) × 100 [25]. Spontaneous aggregation was calculated in all cases.

RESULTS

By flow cytometry in the peripheral blood of these three patients, the CD14+ cells were 15 %, 25%, 22%, respectively, while 1.8%, 0.5%, 3% of them were CD43+. The CD45+ were 98%, 99.5%, 98.5%, respectively, while 3.8%, 6%, 9.9% of them were CD43+. The CD33+ were 0.5%, 1%, 0.3%, respectively, while 0%, 0%, 0% were CD43+. The CD56+ were 8%, 12%, 9%, respectively, while 2%, 3.5%, 6.5% of them were CD43+. The CD34+ cells were 2%, 1.5%, 2.8%, respectively, and 3%, 5%, 4.6% of them were CD43+.

In the studied patients the CD43(+) cells were found in very low percentage in the mononuclear population (lymphocyte and monocytic population) from peripheral blood and bone marrow (3.8%, 6%, 9.9%).

To determine whether this phenomenon is due to downregulation of the sialophorin gene or cleavage and/or internalization of the antigen from the surface we used RT-PCR and western blot analysis to detect the amount

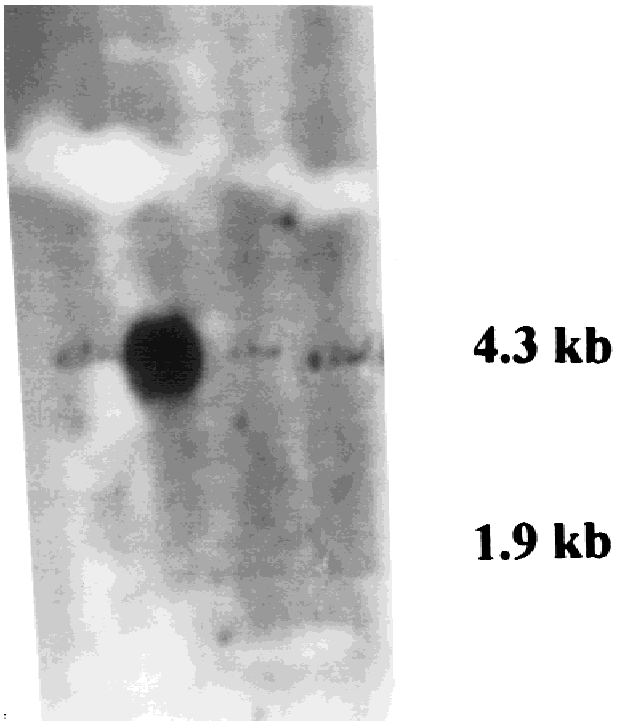

Fig. 3. Northern blot analysis from MDS patients. RNA was extracted from total bone marrow mononuclear cells (BMMNC). Equal amount of RNA was loaded in each lane as it is estimated from the intensity of 18S band. The blots were hybridized with an 187 bp CD43 probe biotinylated. Lanes 1, 3, 4: total RNA from BMMNC from the three patients. Lane 2: CD43(+) cells isolated with magnetic separation. In this particular case CD43+ cells express clonally only the 4.3 bp transcript.

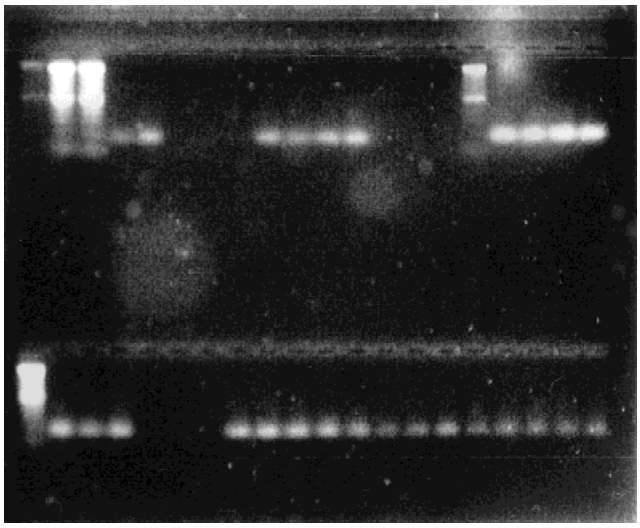

Fig. 4. RT-PCR analysis from the patients with MDS. Lanes 20, 25, 37, 38: 100 bp DNA ladder. Lanes 1–12, 17–23, 29–31, 35, 36: MDS patients without CD43(−) population in the BMMNC. Lanes 13, 24, 32: CD43(+) BMMNC from patients A, B, and C, respectively. Lanes 14, 26, 33: CD43(−) BMMNC from patients A, B, and C, respectively. Lanes 15, 27, 34: total BMMNC from patients A, B, C. Lanes 16, 28, 35: CD34(+) cells from patients A, B, C. A primer pair was used that gave a CD43 product of 187 bp.

of the RNA and the protein produced. We found very small amounts of RT-PCR products and very small amounts of sialophorin mRNA by northern blot as well as very small amounts of sialophorin protein in the protein extracts of the cells of these patients [Figs. 1–4].

We isolated pure CD43 negative and CD43 positive cells from the BMMNC and PBMNC of these patients. We did not detect sialophorin mRNA and sialophorin protein in the protein extracts of CD43(−) cells. The RT-PCR from these cells did not give any product compared with the CD43(+) cells that gave strong RT-PCR product and strong western blot signal [Figs. 1–4].

To investigate whether the reduced production of the CD43 mRNA was related to an acquired mutation of the sialophorin gene SSCP analysis and sequence was performed. No difference was found between the gene sequence of CD43(+) and CD43(−) cells at the same patient.

The total CD34(+) cells from these three patients were subjected to RT-PCR analysis and gave very small amplification product of the sialophorin gene [Fig. 2] compared to the same cell population of the patients with high CD43 expression . The western blot from these cells gave very small signal for the CD43 antigen [Fig. 1].

In order to see the pattern of CD43 distribution in the bone marrow of these patients, we studied paraffin sections and found very few CD43+ cells in the hemopoietic precursors in these three patients.

The CD43 antigen is known to be involved in the aggregation when stimulated with anti-CD43 antibody. We tried to quantitate this phenomenon in the three patients and the different type of cells studied. We found that the aggregation phenomenon was strong after stimulation with the DF-T1 antibody in the CD43+ isolated population, and did not differ from spontaneous aggregation in CD43(−) cells and in BMMNC of the above three patients (Fig. 6).

DISCUSSION

CD43 has been shown to cover up to 19% of thymocytes cell surface [3]. The extended rodlike extracellular conformation of the molecule may result in sterical hindrance of adhesion receptors or ligands. The accessibility to these membrane structures could be drastically modified by shedding of 40–80% of CD43 molecule during cell activation [3,26]. Earlier studies have proved that CD43, when expressed by opposing cells, functions as an anti-adhesion molecule. Because LFA-1 mediated adhesion constitutes a dominant pathway among lymphocytes [27–30] it is suggested that CD43 is physically associ-

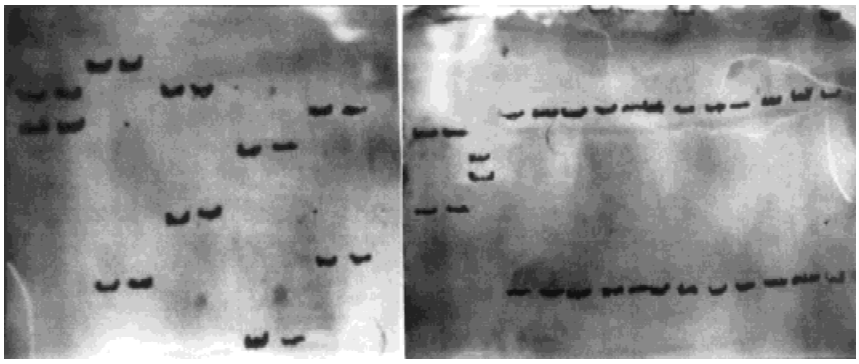

Fig. 5. SSCP analysis: various portions of the sialophorin gene amplified from the CD43(+) cells and CD43(−) cells run close together in pairs. Lanes 14–21: 1st exon amplified using flanking primers from DNA of total bone marrow cells from patients with high expression of CD43 gene. Lanes 22–25: same exon from DNA of normal individuals.

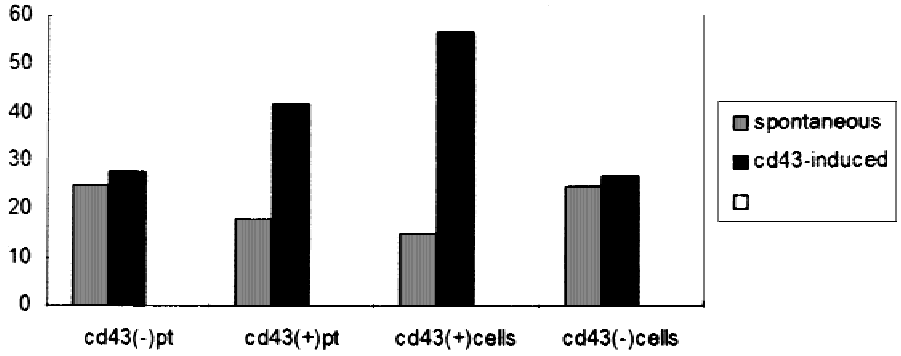

Fig. 6. In vitro aggregation after anti-CD43 stimulation of cells for 72 h. CD43(−)*pt:* bone marrow cells from patient with low expression of CD43 antigen. CD43(+)*pt:* bone marrow cells from patient with high expression of CD43 antigen. CD43(+) *cells:* CD43 positive cells after magnetic isolation. CD43(−) *cells:* CD43 negative cells after magnetic depletion.

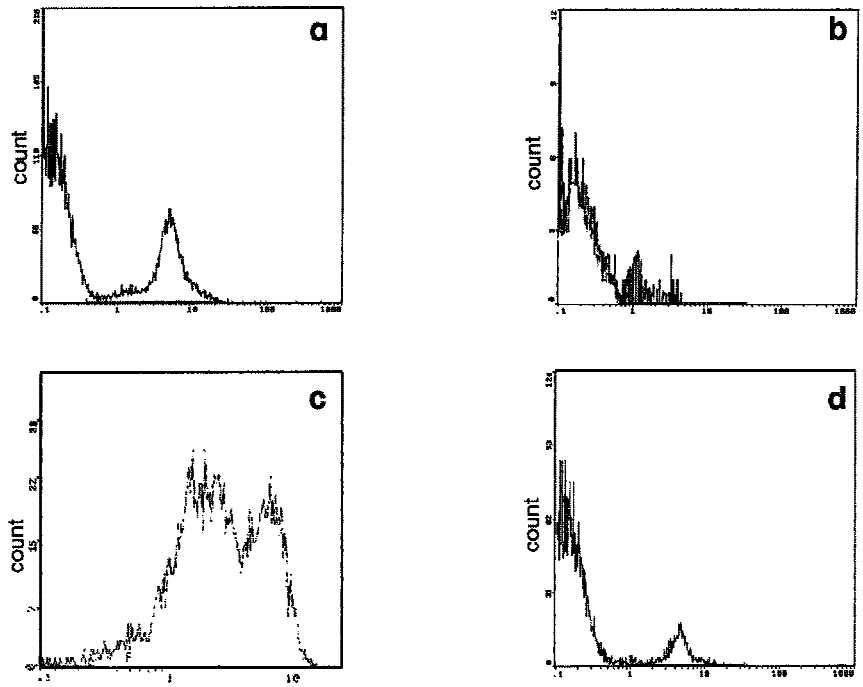

Fig. 7. Flow cytometer fluorescence curves (a), (b), and (d) from patients with MDS (RAEB-t) with low CD43 expression (peripheral blood mononuclear cells). The first curve close to the *y* axis is background. In (c) is a curve from a patient with MDS (RAS) and normal CD43 expression. The curve in the peripheral blood mononuclear cells is usually biconcave in normal population, and the background curve is not seen.

ated with a T-cell receptor complex and may modify T-cell activation through a costimulatory pathway independed of CD28 [28]. Another possible function is its involvement in a macromolecular complex of protein expressed on monocytes together with LMP2 related to C1q binding [29].

Its co-localization with ezrin/radixin/moesin family members means that it may determine cellular activities

such as movement, cell division, cell adhesion, anchorage, and microvilli formation [31]. Through an unknown mechanism it is involved in reduction in susceptibility to G1 arrest and retardation to the apoptotic process in WEHI cell line [27].

In summary the known functions of CD43 are [32] anti-adhesion, adhesion, cell signaling, and cytoskeletal interactions. The anti-adhesion property is due to the extracellular conformation and electrical charges. The level of expression establishes a threshold that adhesive forces must overcome to promote cell interactions. The adhesive function originates from the interaction of CD43 with its receptor or through enabling other ligand–receptor interactions. CD43 ligand–receptor interaction can initiate cell signaling pathways. Cd43 itself includes three sites of protein kinase C phosphorylation. In addition its interaction with cytoskeletal proteins can either remove the extracellular charge barrier or increase the potential for CD43 ligand–receptor interaction.

In neutrophils it has been reported to be related to cell activation and may thus allow previously hindered interaction by exposing new adhesion molecules [26]. Disturbance in the CD43 expression have been recognized in Wiscott Aldrich syndrome [33]. In an effort to identify acquired disorders of cell surface adhesion molecules in patients with myelodysplastic syndromes (MDS) we indentified 3 patients: one with RAEB and 2 with RAEB-t that CD43 was downregulated in peripheral and bone marrow mononuclear cells. This phenomenon was proved at protein and RNA level as it is suggested by western and northern blot analysis and by RT-PCR. When we isolated CD43(+) and CD43(−) cells from these patients there was no mutation found associated with the CD43(−) population of these patient. The remaining 29 patients studied expressed the CD43 antigen at high levels and the percentage of CD43 positive cells was high (50–100%). RT-PCR from these patients gave strong PCR product. The lymphocyte subsets did not differ significantly from those of the normal individual in the pattern and intensity of CD43 expression (data not shown). The expression of CD43 in the neutrophils of the three patients reported was very low while in all the other MDS patients studied was high (40–100%) as shown by flow cytometry.

There are some possible explanations of this phenomenon associated with the regulation of the expression of this gene. It has been described that downregulation of CD43 gene is done through DNA methylation. Possible disturbance of methylation mechanism and methyltransferase system should be studied. It is also known that Sp1 acts on the promoter of sialophorin gene. This transcription factor and its action should be studied, too.

Another possibility is the action of other regulatory elements acting on the above or other mechanisms affecting gene expression.CD43 underexpression has been described to be associated with increased spontaneous aggregation in some cell lines [34]. This phenomenon was not detectable in PBMNC of our patients. The spontaneous aggregation did not differ significantly while the anti-CD43 induced aggregation was significantly lower in the three reported cases.

It is known that in the MDS syndromes one or more clones of dysplastic cells can be recognized together or without normal hemopoietic cells at least in some stages of the disease progress. Usually the dysplastic clone spares the lymphcyte progenitors. In a future study we will try to answer whether the downregulation of the gene is restricted to the pre-leukemic cells or not and which of the hemopoietic progenitors display the phenomenon.

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